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Structural elucidation and antioxidant activities of exopolysaccharides from *Lactobacillus helveticus* MB2-1

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ABSTRACT

In the present study, the crude polysaccharides (EPS) from *Lactobacillus helveticus* MB2-1 were fermented with reconstituted whey medium and further fractionated on DEAE-52 cellulose and Sephadex G-100 chromatography to afford three purified fractions of EPS-1, EPS-2 and EPS-3. Then, their preliminary structures and antioxidant activities *in vitro* were investigated. Three purified EPS fractions (EPS-1, EPS-2 and EPS-3) had similar molecular weight distributions (about 2×10^5 Da), and it was composed of galactose, glucose and mannose with a molar ratio of 1.33:2.75:1.00, 1.00:1.43:9.34 and 1.17:1.00:2.96, respectively. In addition, the antioxidant activity of EPS-1, EPS-2 and EPS-3 was evaluated with the *in vitro* scavenging abilities. The results indicated that both crude and purified EPS showed strong scavenging activities on three kinds of radical and chelating activities on ferrous ion, and their antioxidant activities decreased in the order of crude EPS > EPS-3 > EPS-2 > EPS-1.

1. Introduction

There are increasing evidences indicating that excessive production of reactive oxygen species (ROS) and oxygen-derived free radicals may play an important role in tissue damage and loss of function in many tissues and organs. Some ROS may also damage cell membranes and DNA, inducing many diseases including inflammation, diabetes, atherosclerosis, aging and cancer (Chen, Zhang, Jiang, Mu, & Miao, 2012; Wang, Mao, & Wei, 2012; Wang, Zhang, Wang, & Wang, 2013). In order to reduce ROS-induced oxidative damage, both synthetic and natural antioxidants are used. However, there are doubts about the safety and long-term effects on health of synthetic antioxidants. For instance, the commonly used synthetic antioxidants such as butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) are suspected to have some toxic effects and as possible carcinogens (Mao, Yin, Ge, Jiang, & Gong, 2013; Yang et al., 2012; Zhang et al., 2012). Therefore, it is desirable to develop natural nontoxic antioxidants with possible antioxidant and/or radical scavenger properties as alternatives to synthetic ones in pharmaceutical and food industries. By far, it has been reported that some polysaccharides from plants, animals, yeast and microorganisms (fungi and bacteria) possessed *in vitro* antioxidant activities (Kodali & Sen, 2008; Liu et al., 2009; Qiao et al., 2009).

Among microbial polysaccharide, those exopolysaccharides (EPS) produced by lactic acid bacteria (LAB) are receiving increasing attention because these microorganisms have food-grade status (Axelsson, 2004; Charchoghlyan & Park, 2013). Those EPS have been reported with unique physical and rheological properties that facilitate their applications in the fermented dairy industry because of their potential application as viscosifiers, emulsifying agents and texturizers (Chiang & Pan, 2012; Coda, Lanera, Trani, Gobbetti, & Cagno, 2012). EPS producing LAB has been broadly used to improve the rheology, texture and mouthfeel of fermented milk products such as yogurt and cheese. In addition, there has been an increasing interest in exploiting the EPS-producing LAB for their possible biological activities including antitumor, immunostimulatory, cholesterol lowering activity and antioxidant activity (Liu et al., 2011; Pan & Mei, 2010; Suresh et al., 2013; Yang et al., 2012). Therefore, EPS from LAB has potential for development as food additives or functional food ingredients with both health and economical benefits.

Lactobacillus helveticus, an obligately heterofermentative LAB, plays an important role in the dairy industry as starter microorganism for the manufacture of fermented milk and semi-hard cheeses (Johnson-Henry, Hagen, Gordonpour, Tompkins, & Sherman, 2007; Vinderola, Matar, Palacios, & Perdigón, 2007). In recent years, an increased interest has been focused on ropy strains for the production of yogurt and Mozzarella-like cheeses since the EPS synthesized enhances the viscosity and extensibility of these products. In previous reports, the ropy strain *L. helveticus* ATCC 15807

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is able to synthesize a high molecular mass EPS (1.8×10^6 Da) composed of galactose and glucose (1:2) when grown in milk cultures (Torino, Mozzi, Sesma, & Valdez, 2000). This microorganism produces higher amounts (549 mg/mL) of EPS under acidic culture conditions when grown at 37 °C in milk (Torino, Taranto, Sesma, & De Valdez, 2001). We also noted that *L. helveticus* MB2-1 could produce high amount and viscosity EPS (Li, Mutuvulla, Chen, Jiang, & Dong, 2012). Till now, there have a few reports on the biosynthesis, purification and characterization of EPS from *L. helveticus* (Lin & Chien, 2007; Staaf, Yang, & Widmalm, 2000; Torino, Mozzi, & de Valdez, 2005; Yang, Staaf, & Widmalm, 2000). However, to our knowledge, the antioxidative effect of EPS produced from *L. helveticus in vitro* has not been reported yet. Therefore, evaluation of the antioxidant activities of EPS produced by *L. helveticus* will be important for the elucidation of function and utilization of the EPS.

In this paper, we describe the production, isolation, purification, characterization and antioxidant potential of EPS derived from *L. helveticus* MB2-1 that isolated from traditional Sayram ropy fermented milk (SRFM) in southern Xinjiang of China.

2. Materials and methods

2.1. Materials and reagents

1,1-Diphenyl-2-picryl-hydrazyl (DPPH), nitro blue tetrazolium (NBT), phenazine methosulfate (PMS), dihydromicotineamidadenine dinucleotide (NADH), Vitamin C (Vc), 1,10-phenanthroline, ferrozine, rhamnose, arabinose, fucose, xylose, mannose, glucose, galactose and inositol were purchased from Sigma Chemical Co. (St. Louis, MO, USA). DEAE-cellulose 52, Sephadex G-100 and T-series dextrans were purchased from Whatman Co. (Maidstone, Kent, UK) and Pharmacia Co. Ltd (Uppsala, Sweden), respectively. All other reagents were of analytical grade.

2.2. Strains and medium

L. helveticus MB2-1 was isolated from traditional SRFM, which was collected from southern Xinjiang, China (Li et al., 2012). It was identified according to its morphological characteristics and 16S rDNA sequences analysis. Cultures were stored at −20 °C in 10% (w/v) sterile reconstituted skim milk containing 0.5% (w/v) yeast extract, 1.0% (w/v) glucose and 20% (v/v) glycerol.

Whey powder was obtained from Great Lakes Cheese of New York Inc. (Adam, New York, USA). Whey was reconstituted with distilled water to 8% (w/v) final concentration containing 0.5% (w/v) yeast extract, and the pH was adjusted to 6.7 with 2.0 M NaOH. The reconstituted whey was heat treated at 110 °C for 20 min, stored at 4 °C (no longer than 2 days) and used as fermentation medium. The composition (%, w/w) of the whey powder employed in this study was the following: fat, 1.0; protein, 2.5; moisture, 5.0; lactose, 80.0; and sodium salts, 0.4.

2.3. Analysis of bacterial growth and EPS production

Cultivations of *L. helveticus* MB2-1 for EPS production were performed as batch cultures in 800 mL of reconstituted whey at 40 °C in 2000 mL Erlenmeyer flasks. Samples (50 mL) were withdrawn at regular intervals to determine pH with a Schott pH Meter (model, Lab 850, Mainz, Germany), number of microbial viable counts, optical density (OD) values and yields of EPS. The viable counts were determined by dilution plating with MRS agar medium incubated at 37 °C for 48 h. The biomass growth of the *L. helveticus* MB2-1 was estimated by measuring the optical density (OD) of the culture at 600 nm with a 722 spectrophotometer (Shanghai Analytical instrument factory, Shanghai, China). EPS was isolated from culture sample by the described as Section 2.4. The EPS contents were determined by the modified phenol–sulfuric acid method using glucose as a standard (Li et al., 2009).

2.4. Isolation and purification of EPS

The EPS was isolated according to our previously method (Li et al., 2012). Briefly, after 24 h of incubation period, the cell was separated by centrifugation at 12,000 g for 15 min at 4 °C. Then trichloroacetic acid (TCA) solution was added to the culture to give a final concentration of 4% (w/v), and the precipitated proteins were removed by centrifugation (12,000 × *g* for 30 min at 4 °C). The supernatant was filtered through a 0.45 μm membrane filter, mixed with three volumes ice cold ethanol, stirred vigorously and kept at 4 °C for overnight. Crude EPS was collected by centrifugation at 15,000 × *g* for 15 min. The EPS pellet was dissolved in distilled water and dialyzed (Mw cut-off 8000–14,000 Da, Solarbio Co., Ltd, Beijing, China) against same solution for 48 h against distilled water at 4 °C and then lyophilized.

The freeze-dried sample was fractionated with an anion exchange chromatography on the DEAE-cellulose column (2.6 cm × 30 cm), eluted with a step gradient of NaCl solution (0, 0.1 and 0.3 M) at a flow rate of 60 mL/h. The fractions were assayed for carbohydrate content by the phenol–sulfuric acid method. Peak fractions containing polysaccharides were pooled, dialyzed and lyophilized. Further purification of the EPS was performed by gel filtration using a Sephadex G-100 column (2.6 cm × 100 cm) eluted with distilled water at a flow rate of 12 mL/h. The major polysaccharide fraction was pooled, dialyzed with water and freeze-dried for further study.

2.5. Chemical composition analysis

Total sugar content was determined by the phenol–sulfuric acid method as mentioned above (Li et al., 2009). Protein content was assessed as described by Bensadoun and Weinstein (1976). Uronic acid content was measured as described by Blumenkrantz and Asboe-Hansen (1973). Sulfate group content was determined according to Silvestri, Hurst, Simpson, and Settine (1982).

2.6. Determination of purity and molecular weight

Purity and molecular weight (Mw) were determined by high-performance size-exclusion chromatography (HPSEC) on an Agilent 1200 system (Palo Alto, CA, USA) equipped with a TSK gel G4000 PW XL column (300 mm × 7.8 mm, Tosoh Corp., Tokyo, Japan) and an evaporative light scattering detector (ELSD) (Yang et al., 2012). Sample solution (20 μL) was injected and eluted with distilled water at 50 °C with a flow rate of 0.7 mL/min. The linear regression was calibrated with T-series dextrans standards (T-500, T-200, T-100, T-50, and T-10).

2.7. Spectra analysis

Ultraviolet–visible (UV–vis) spectroscopy analyses were conducted on UV–vis spectrophotometer (U-4100, Hitachi Ltd., Japan). The EPS solution was prepared by suspending the sample in distilled water to a concentration of 1.0 mg/mL for UV–vis measurement in the wave-length range of 190–500 nm. Fourier-transform infrared (FT-IR) spectra were recorded from the sample in KBr pellet on a FT-IR spectrophotometer (NICOLET NEXUS470, Thermo Nicolet Co., WI, USA). In order to obtain more exact band positions, all the FT-IR analysis of EPS fractions were performed in the region 400–500 cm^{-1}.

2.8. Analysis of monosaccharide composition

Monosaccharide composition of different purified EPS fractions was determined by Gas chromatography (GC) as described by Liu et al. (2009). Briefly, 5 mg of polysaccharide was hydrolyzed with 2 mL of 2 M trifluoroacetic acid (TFA) at 120 °C for 2 h. The hydrolyzate was repeatedly co-concentrated with methanol to dryness and converted to its trimethylsilyl (TMS) derivative. The TMS derivative of hydrolyzate was prepared by adding 0.2 mL trimethylchlorosilane, 0.4 mL hexam-ethyldisilazane and 1 mL pyridine and heating at 80 °C for 30 min. Seven standard sugars (rhamnose, arabinose, fucose, xylose, mannose, glucose and galactose) and internal standard (inositol) were converted to their TMS derivatives in the same way. After cooling, samples were analyzed on HP 6890 GC (Agilent Technogolgies, Palo Alto, CA, USA) equipped with flame ionization detector (FID) and a HP-5 fused silica capillary column (30 m × 0.32 mm × 0.25 mm, J&W Scientific Inc., Folsom, CA, USA). The following chromatographic conditions were used: N_2 was used as the carrier gas at a flow rate of 1 mL/min; the temperature of injector and detector were set at 250 °C and 280 °C, respectively; initial column temperature was held at 100 °C for 5 min, then programmed at a rate of 5 °C/min to 150 °C and held at 150 °C for 5 min, subsequently programmed at 5 °C/min to 240 °C and held at 240 °C for 2 min. Calculation of the molar ratio of the monosaccharide was carried out on the basis of the peak area of the monosaccharide.

2.9. Antioxidant activity tests

2.9.1. DPPH free radical scavenging activity

The DPPH free radical scavenging activity was measured by using the method reported by Qiao et al. (2009) with slight modification. Briefly, 0.2 mL ethanolic DPPH radical solution (0.4 mM) was added to 1.0 mL sample solution (0.125–4 mg/mL), and then 2.0 mL of deionized water was added. The mixture was shaken and allowed to stand at room temperature in the dark for 30 min. The mixture was mixed vigorously and incubated at room temperature in the dark for 30 min. The absorbance was measured at 517 nm against a blank. Lower absorbance of the reaction mixture indicates higher free-radical scavenging activity. The scavenging activity on DPPH radical (%) = $[(1 - (A_{sample} - A_0)/A_{blank})] \times 100$, where A_0 is the absorbance of the sample under identical conditions as A_{sample} with water instead of DPPH radical solution. Deionized water and Vc were used as the blank and positive control, respectively.

2.9.2. Hydroxyl radical scavenging activity

The hydroxyl radical scavenging activity was investigated with the method mentioned by of Liu et al. (2009). The hydroxyl radical was generated in the mixture of 1 mL of 0.75 mM 1,10-phenanthroline, 1 mL of 0.75 mM $FeSO_4$, 1 mL of H_2O_2 (0.01%, v/v) and 1.5 mL of 0.15 M sodium phosphate buffer (pH 7.4). After addition of 1.0 mL sample solution (0.125–4 mg/mL), the mixture was incubated at 37 °C for 30 min. The absorbance of the mixture was measured at 536 nm. The scavenging activity on hydroxyl radical (%) = $(A_{sample} - A_{blank})/(A_0 - A_{blank}) \times 100$, where A_0 was the absorbance of the deionized water instead of H_2O_2 and sample in the assay system. Deionized water and Vc were used as the blank and positive control, respectively.

2.9.3. Superoxide anion scavenging activity

The superoxide radical scavenging activity was conducted according to Liu et al. (2009) with some modifications. The superoxide radical was generated in 3 mL of 0.1 M sodium phosphate buffer (pH 7.4) containing 156 μM NADH, 52 μM NBT and 20 μM PMS. After addition of 1.0 mL sample solution (0.125–4 mg/mL), the mixture was incubated at 25 °C for 5 min. The absorbance of the mixture was measured at 560 nm. The scavenging activity on superoxide radical (%) = $(1 - A_{sample}/A_{blank}) \times 100$. Deionized water and Vc were used as the blank and positive control, respectively.

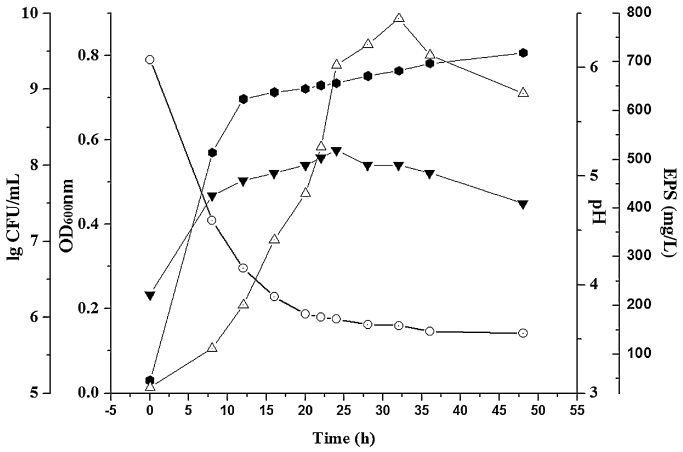

Fig. 1. Kinetics of growth and EPS production by *L. helveticus* MB2-1 in reconstituted whey medium at 37 °C showing the biomass growth (●), viable cell counts (▼), pH (○) and amounts of EPS produced (△).

2.9.4. Metal ion chelating activity

The chelating ability of metal ion was measured according to the method reported by Qiao et al. (2009) in terms of chelating ferrous ion (Fe^{2+}) in the iron–ferrozine complex. Briefly, the reaction mixture, containing 1.0 mL sample solution (0.125–4 mg/mL), 0.05 mL ferrous chloride ($FeCl_2$) solution (2 mM), 0.2 mL ferrozine solution (5 mM) and 2.75 mL water, was shaken well and incubated at room temperature for 10 min. The absorbance of the mixture was measured at 562 nm against a blank. The chelating ability on ferrous ion (%) = $[(A_{blank} - (A_{sample} - A_0)/A_{blank})] \times 100$, where A_0 is the absorbance of the sample under identical conditions as A_{sample} with water instead of $FeCl_2$ solution. Deionized water and EDTA-Na were used as the blank and positive control, respectively.

2.10. Statistical analysis

All data were analyzed by one-way ANOVA. Tests of significant differences were determined by Turkey-HSD at ($P < 0.05$). In all cases, there were three replicates ($n = 3$).

3. Results and discussion

3.1. Time courses of bacterial growth and EPS production

The time courses of biomass growth, viable counts, pH and accumulation of EPS are shown in Fig. 1. No lag phase was observed when the cells were grown in reconstituted whey medium. The cells entered into the stationary phase at about 12 h and the biomass concentrations in terms OD_{600nm} reached a maximum value of 0.79 at 36 h and remained constant until the end of the experiment at 48 h. In contrast, once the viable cell counts reached their maximum (8.25 lg CFU/mL) after 24 h, the values decreased at the beginning of the declining phase. The formation trend of EPS was similar to that of viable cell counts. Production of EPS by *L. helveticus* MB2-1 reached a maximum at 32 h in the late stationary phase of growth, and EPS was hydrolyzed quickly during subsequent fermentation. The maximum concentration of EPS at 32 h was estimated to be approximately 658 mg/L by phenol-sulfuric acid method using glucose as standard. The decrease in EPS amounts after prolonged incubation was observed earlier for different EPS-producing *Lactobacillus* strains (Zhang et al., 2012), probably due

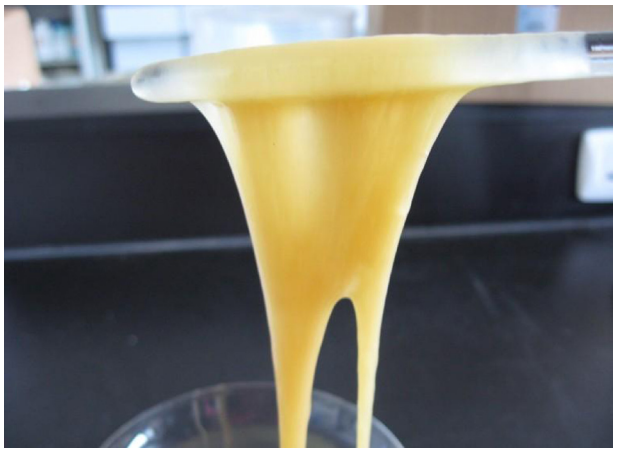

Fig. 2. Photography of the isolated high viscosity crude EPS from reconstituted whey medium.

to the action of glycohydrolases in the culture that catalyzed the degradation of polysaccharides. However, Kodali and Sen (2008) reported that the lack of EPS degradation during the fermentation (up to 56 h) with the probiotic bacterium *Bacillus coagulans* RK-02 was attributed to the lack of glycohydrolase activity in the culture.

3.2. Isolation and purification of EPS

The crude viscosity EPS (Fig. 2) obtained from the treatment with TCA and subsequently with cold ethanol of the growth medium supernatant of *L. helveticus* MB2-1 was fractionated first on an anion-exchange chromatography column of DEAE-52 cellulose based on the difference of ionic groups present in EPS molecule. Three fractions eluted with 0, 0.1 and 0.3 M sodium chloride solutions were collected (F-1, F-2, and F-3), respectively (Fig. 3A). F-1 eluted with distilled water was known as neutral polysaccharides, and F-2 and F-3 eluted with 0.1 M and 0.3 M NaCl solutions were known as acidic polysaccharides (Gan, Ma, Jiang, Xu, & Zeng, 2011). The three fractions were collected, concentrated and purified by gel filtration chromatography of Sephadex G-100, respectively. As a result, each fraction generated one single elution peak (Fig. 3B–D), named as EPS-1, EPS-2 and EPS-3, respectively. Finally, the three purified fractions were collected, concentrated, dialyzed and lyophilized for further study, respectively. The recovery rates of EPS-1, EPS-2 and EPS-3 based on the amount of crude EPS were 28.2%, 39.5% and 2.4%, respectively.

3.3. Contents of carbohydrate, protein, uronic acid and sulfate in EPS

Table 1 shows the contents of carbohydrate, protein, uronic acid and sulfate in crude EPS and three purified fractions. The carbohydrate contents in crude EPS, EPS-1, EPS-2 and EPS-3 were 71.68%, 97.85%, 94.54% and 67.62%, respectively. Among all the EPS tested, the relative higher contents of uronic acid in crude EPS and EPS-3 were 2.98% and 2.53%, respectively. The contents of sulfate in crude EPS, EPS-1, EPS-2 and EPS-3 were 1.43%, 0.27%, 0.42% and 0.87%, respectively. The relatively high content of protein (0.29%) for EPS-3 suggested that it might be protein-bound polysaccharide (Qiao et al., 2009).

3.4. Molecular weight (Mw) and monosaccharides composition of EPS

The HPSEC system was used to determine homogeneity and Mw of the three purified EPS fractions. The homogeneity results

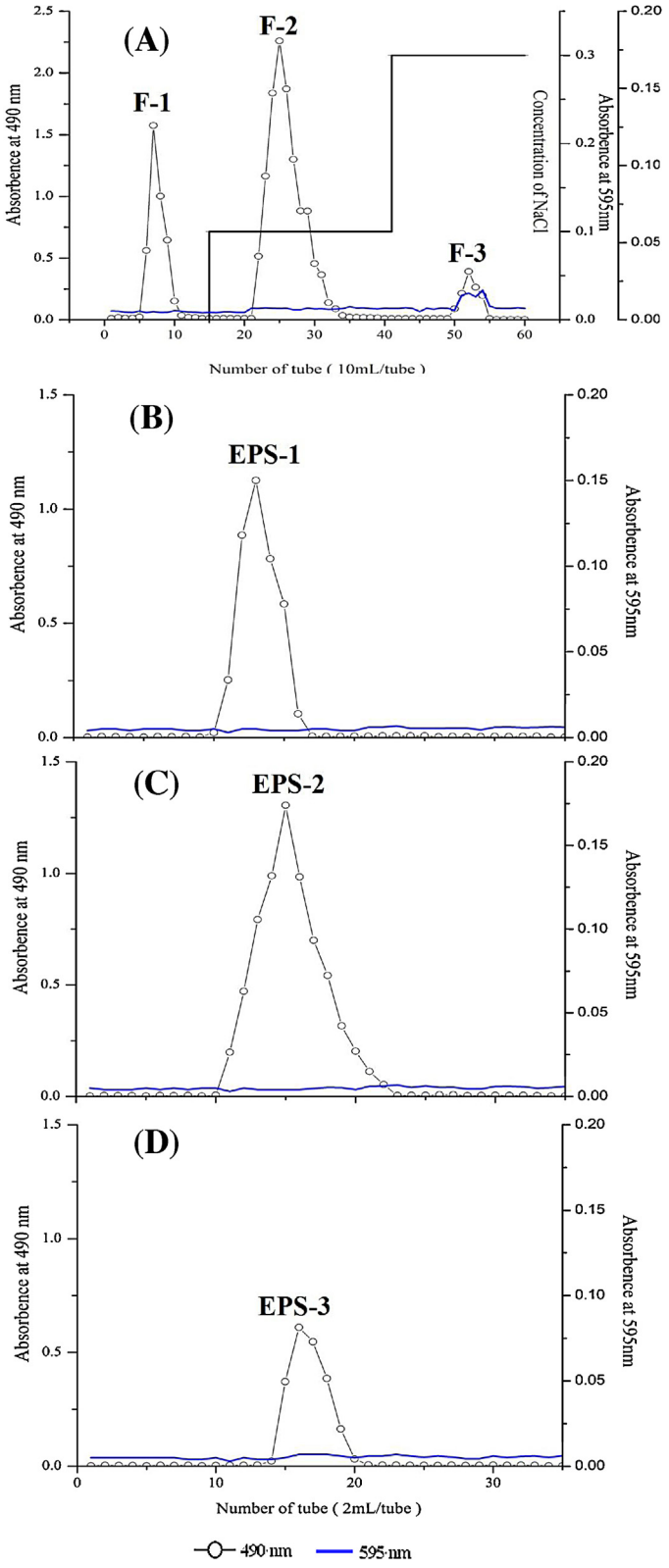

Fig. 3. Elution profile of crude viscosity EPS on DEAE-cellulose chromatography column with gradient of NaCl solution (0, 0.1 and 0.3 M) at a flow rate of 60 mL/h (A); Elution profile of EPS-1 (B), EPS-2 (C) and EPS-3 (D) on Sephadex G-100 gel chromatography column with distilled water at a flow rate of 12 mL/h, respectively. –○– 490 nm for detection of carbohydrate; ▬ 595 nm for detection of protein.

Table 1
The monosaccharides composition and the contents of carbohydrate, protein, uronic acid and sulfate for crude EPS, EPS-1, EPS-2 and EPS-3.

Sample	Carbohydrate (%)	Protein (%)	Uronic acid (%)	Sulfate (%)	Sugar component (molar ratio)[a]		
					Gal	Glc	Man
Crude EPS	71.68	4.08	2.98	1.43	1.00	1.69	3.54
EPS-1	97.85	–[b]	0.53	0.27	1.33	2.75	1.00
EPS-2	94.54	–	1.96	0.42	1.00	1.43	9.34
EPS-3	67.62	0.29	2.53	0.87	1.17	1.00	2.96

[a] Individual components were identified and quantified based on elution of known standards, and data are presented as mol ratio for each sugar.
[b] Not detected.

are shown in Fig. 4A–C. EPS-1, EPS-2 and EPS-3 showed only one symmetrical sharp peak on HPLC, respectively, which indicated that three EPS fractions were the homogeneous polysaccharide. The equation of the standard curve was: Log $M_w = -36.923\,T_R + 508$ (where M_w represents the molecular weight, while T_R represents retention time) with a correlation coefficient of 0.9993. According to the calibration curve of the elution times of standards, the molecular weights of EPS-1, EPS-2 and EPS-3 were estimated to be 2.08×10^5, 2.04×10^5 and 2.01×10^5 Da, respectively, which suggested that three purified EPS fractions have similar molecular weight distributions.

The monosaccharides composition of EPS-1, EPS-2 and EPS-3 was analyzed by GC. Chromatograms of monosaccharides composition of three purified EPS fractions were shown in Fig. 5, and the molar ratio of sugars were summarized in Table 1. The results indicated that all the *L. helveticus* MB2-1 EPS fractions were rich in galactose, glucose and mannose. The molar ratios of galactose, glucose and mannose in EPS-2 and EPS-3 were 1.00:1.43:9.34 and 1.17:1.00:2.96, respectively. The results indicated that mannose were the predominant monosaccharides in EPS-2 and EPS-3. Notably, the molar ratios of monosaccharide (1.33:2.75:1.00) in EPS-1 were quite different from the other two EPS fractions.

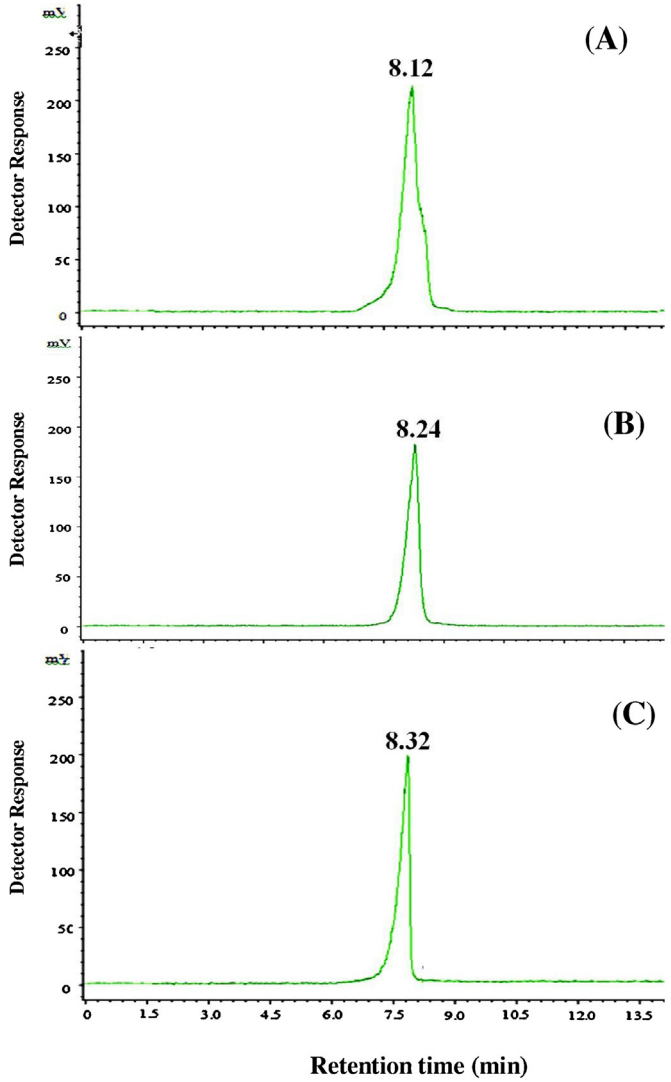

Fig. 4. High-performance size-exclusion chromatography (HPSEC) of EPS-1 (A), EPS-2 (B) and EPS-3 (C).

3.5. Spectra analysis

Apart from crude EPS and EPS-3, UV–vis spectra of the EPS-1, EPS-2 showed only single peak at 210 nm and no other peak was detected in 260–290 nm, which clearly explain that the EPS-1 and EPS-2 did not have any protein and nucleic acid (Fig. 6A). In order to investigate the functional groups of the purified EPS, the FT-IR spectra were recorded at the absorbance mode from 4000 to

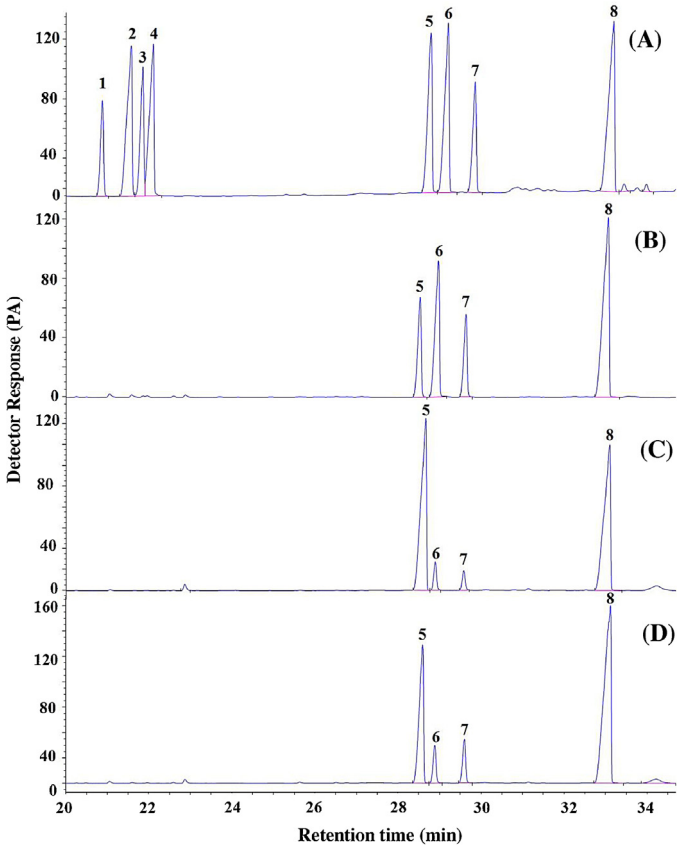

Fig. 5. GC chromatograms of standard (A), EPS-1 (B), EPS-2 (C) and EPS-3 (D) hydrolyzates. The peaks correspond to rhamnose (peak 1), arabinose (peak 2), fucose (peak 3), xylose (peak 4), mannose (peak 5), glucose (peak 6), galactose (peak 7) and inositol (peak 8).

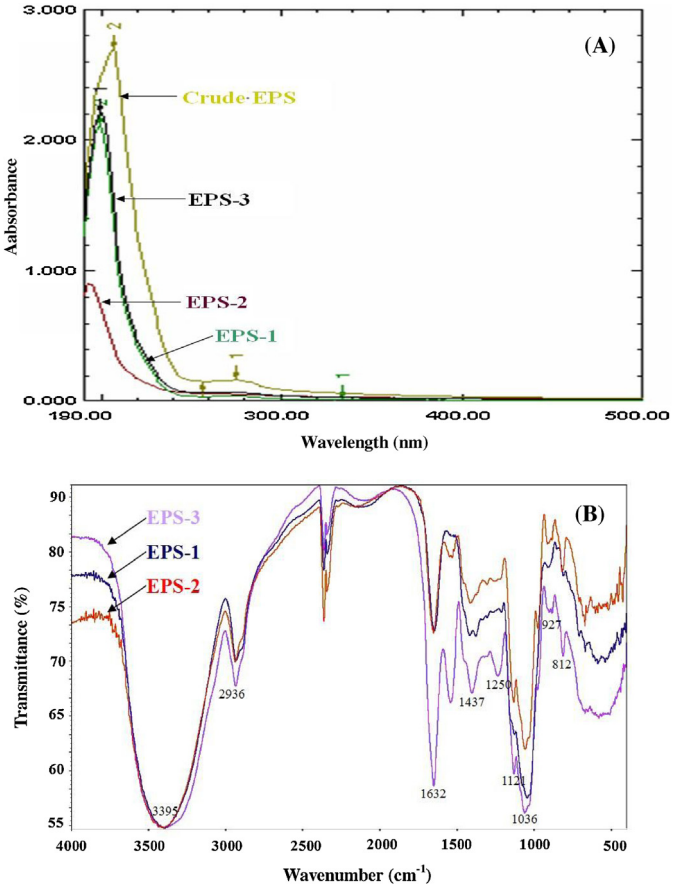

Fig. 6. UV spectra of crude EPS, EPS-1, EPS-2 and EPS-3 in the range 190–500 nm (A); FT-IR spectra of crude EPS, EPS-1, EPS-2 and EPS-3 in the range 400–4000 cm^{-1} (B).

500 cm^{-1}. As shown in Fig. 6B, the spectra of EPS-1, EPS-2 and EPS-3 was displayed an intensity and broad band at around 3395 cm^{-1}, which assigned to the O—H stretching vibration. The peak around 2936 cm^{-1} was due to the C—H stretching vibration (Melo, Feitosa, Freitas, & de Paula, 2002). EPS-1 showed a stronger absorption peak at 2936 cm^{-1} than EPS-2 and EPS-3. The band around 1632 cm^{-1} was due to the bound water (Liu et al., 2009). The bands in the region of 1437 and 1250 cm^{-1} were assigned to C—O stretching vibrations and O—H deformation vibrations (Wang et al., 2012). The absorptions at 1121 and 1036 cm^{-1} attributed to the stretching vibrations of pyranose ring. EPS-1, EPS-2 and EPS-3 were the polysaccharide which contains pyranose ring. Moreover, the band at 927 cm^{-1} indicated the β-pyranose form of the glucosyl residue, and the band at 812 cm^{-1} suggested the β-pyranose form of the mannose residue (Ye, Liu, Wang, Wang, & Zhang, 2012). As might be expected, the FT-IR spectra of EPS-2 and EPS-3 were similar, but different from that of EPS-1, consistent with the differences in molar ratios of monosaccharide. What's more, the relative stronger absorption peak at 1632 cm^{-1} for N—H bending vibration might be related to relatively high content of protein in EPS-3 (Qiao et al., 2010). Therefore, the FT-IR spectra analysis suggested that it was highly likely that the EPS belonged to β-type heteropolysaccharides with pyranose saccharides.

3.6. Antioxidant activity of crude EPS and purified EPS fractions

3.6.1. DPPH free radical scavenging activity

DPPH free radical is a stable radical with an unpaired valence electron at one atom of nitrogen bridge, which decreases significantly on exposure to proton radical scavenger (Kodali & Sen, 2008). On interacting with DPPH free radical, antioxidants transfer either an electron or a hydrogen atom to DPPH, thus neutralizing its free radical character (Liu et al., 2011). The DPPH free radical scavenging activity of crude EPS and three purified EPS fractions was measured in the concentration range 0.125–4 mg/mL by using the colorimetric assay, with Vc as the positive control. As shown in Fig. 7A, scavenging abilities of EPS on inhibition of the DPPH radical were related to the concentration of the samples. The higher concentration, the higher level of scavenging ability was found for all samples used in the test. And the scavenging abilities of the crude EPS was higher than that of its purified fractions (EPS-1, EPS-2 and EPS-3). This was probably due to the presence of other antioxidant components in the crude EPS, such as proteins, peptides and microelements. Among these antioxidant components, there may be some synergistic effects and interactions for antioxidant properties. At 0.125 mg/mL, the three purified EPS fractions showed scavenging abilities of 18.42–22.95% on DPPH free radical. At 4 mg/mL, the scavenging effects increased to 33.53–41.92%. Moreover, the scavenging effect of EPS-3 was stronger than that of EPS-1 and EPS-2 in the same concentration. The differences of the scavenging ability of the three EPS fractions s on DPPH radicals may be due to their chemical features discrepancy. EPS-3 could be better advantageous than EPS-1 and EPS-2 for reacting with DPPH radicals to convert them to more stable products and thereby terminate radical chain reactions. It was postulated that the antioxidant activity of polysaccharides may be associated with Mw, that is, the smaller Mw, the stronger antioxidant activity (You et al., 2013). In the present study, EPS-3 had a smaller Mw than that of the EPS isolated from other LAB (Pan & Mei, 2010; Zhang et al., 2012), which possibly resulted in its strong antioxidant activity. Tsiapali et al. (2001) also stated that the free radical scavenging activity was partially related to monosaccharide composition and glycosidic linkage. In addition, at 0.125–4 mg/mL, the scavenging abilities of Vc on DPPH free radicals were in the range 82.22–89.31%.

3.6.2. Hydroxyl radical scavenging activity

Among the reactive oxygen species, hydroxyl radicals were the most reactive radical and can induce oxidative damage to adjacent biomolecules, which results in aging, as well as cancer and other diseases. Hydrogen peroxide molecules can lead to oxidative injury in the biomolecules indirectly by producing hydroxyl radical *via* fenton reaction and/or iron-catalyzed Haber–Weiss reaction, which can be prevented and/or inhibited by antioxidants (Liu et al., 2009). Thus, removing hydroxyl radical is important for antioxidant defense in cell or food systems (Huang, Ding, & Fan, 2012). The hydroxyl radical, generated by the fenton reaction in the system, was scavenged by the crude EPS, EPS-1, EPS-2, EPS-3 and Vc. The scavenging effects of EPS and Vc are shown as in Fig. 7B. The scavenging abilities of EPS and Vc on hydroxyl radicals were in a concentration-dependent way. The scavenging effects of crude EPS, EPS-1, EPS-2 and EPS-3 increased significantly ($P < 0.05$) with the increase of sample concentration ranging from 1 to 3 mg/mL. After that, the scavenging ability increased slowly with the increase of EPS concentration. At a concentration of 4 mg/mL, the abilities of EPS-1, EPS-2, EPS-3, crude EPS and Vc to scavenge hydroxyl radicals were 38.40%, 56.30%, 62.93%, 80.24% and 97.72%, respectively. The results demonstrate that EPS *L. helveticus* MB2-1 have strong scavenging activity on hydroxyl radical. The EPS minimized the concentration of ferrous ion in the fenton reaction, and the scavenging effects of the EPS might be due to the active hydrogen donating ability of hydroxyl substitutions of EPS. The crude EPS exhibited a stronger scavenging effect against hydroxyl radical than EPS-1, EPS-2 and EPS-3, which was in accordance with the results in Section 3.6.1. However, the scavenging abilities of the crude EPS and

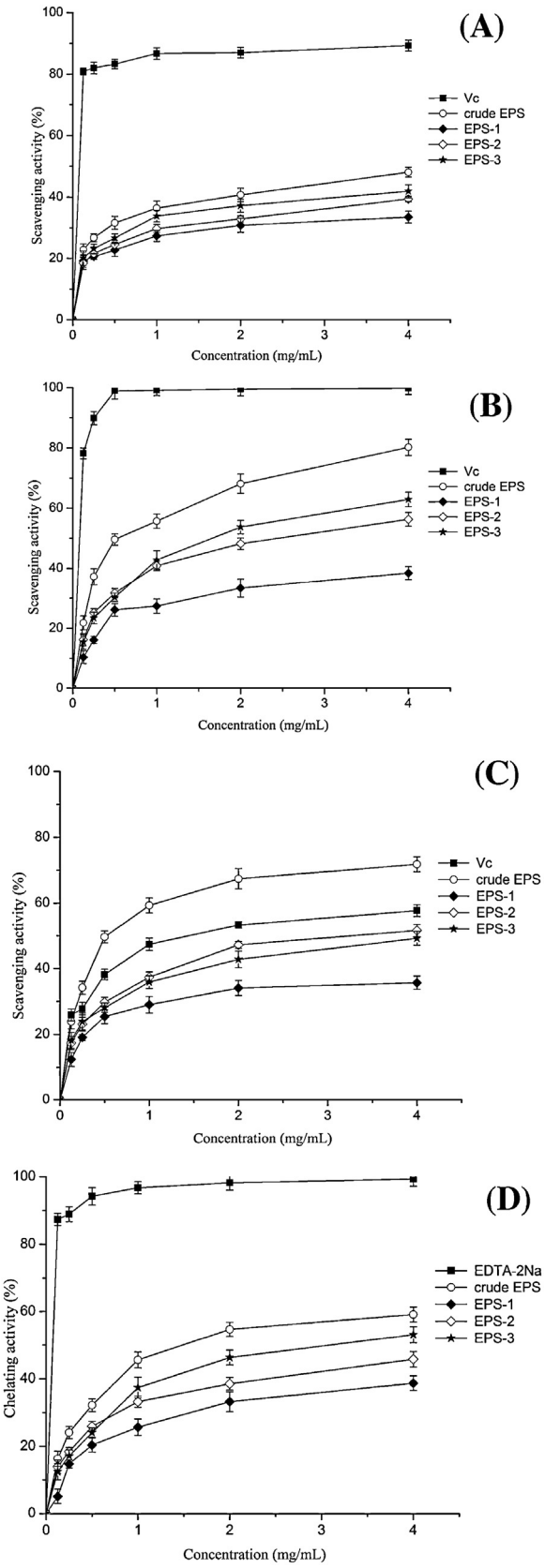

Fig. 7. Scavenging activities on DPPH radical (A), hydroxyl radical (B), superoxide radical (C) and metal ion chelating activity (D) of crude EPS, EPS-1, EPS-2, EPS-3 and positive control. Data are presented as means ± SD of triplicates.

three purified EPS fractions on hydroxyl radicals were all relatively lower than that of Vc at the same concentrations.

3.6.3. Superoxide anion scavenging activity

Among various ROS, superoxide anion, is a precursor to active free radicals that have potential of reacting with biological macromolecules and thereby inducing tissue damage (Mao et al., 2013). Superoxide radicals would play important roles in the formation of other ROS, such as hydroxyl radical, hydrogen peroxide and singlet oxygen, which induce oxidative damage in DNA, proteins and lipids (Liu et al., 2011). Therefore, it was of great importance to characterize the superoxide radical-scavenging potential of different antioxidants. Fig. 7C shows the scavenging activities *in vitro* on superoxide anion of crude EPS, EPS-1, EPS-2, EPS-3 and Vc. Notably, all the EPS samples possessed superoxide anion scavenging activities, especially crude EPS and EPS-2 showed relative stronger activities. At a concentration of 4 mg/mL, the scavenging activities on superoxide anion for crude EPS, EPS-2, EPS-3, EPS-1 and Vc were 71.82%, 51.60%, 49.26%, 35.73% and 56.35%, respectively. In addition, the tested EPS samples were able to scavenge the superoxide radicals in a concentration-dependent manner. Moreover, the scavenging ability of EPS-2 was similar to that of Vc at 4 mg/mL. These results suggested that EPS-2 was an effective scavenger for superoxide radicals, and it might be advantageous for preventing injury induced by superoxide radicals in pathological conditions. Previous study illustrated that the mechanism of scavenging superoxide anion may be associated with dissociation energy of O—H bond. The higher the number of electron-withdrawing groups attached to polysaccharide, the weaker the dissociation energy of O—H bond (Tsiapali et al., 2001). Huang et al. (2012) reported about the antioxidant activity of one neutral polysaccharide and four acidic polysaccharides *in vitro*, and the effective scavenging superoxide anion was attributed to the presence of some types of the electrophilic groups like aldehyde or keto to facilitate liberation of hydrogen from O—H bond, which was associated with superoxide anion scavenging.

3.6.4. Metal ion chelating activity

Metal chelating activity is claimed as one of antioxidant mechanisms, since it reduces the concentration of the catalyzing transition metal in lipid peroxidation (Xu, Ye, Sun, Tu, & Zeng, 2012). Among the transition metals, ferrous ion is known as the most important lipid oxidation prooxidant due to its high reactivity. It was reported that chelating agents, which form s-bonds with metal, are effective as secondary antioxidants because they reduce the redox potential thereby stabilizing the oxidized form of the iron ion (Duh, Tu, & Yen, 1999). Fig. 7D shows the ferrous ion chelating activities of crude EPS, EPS-1, EPS-2, EPS-3 and EDTA-Na. Ferrous ion could be combined by all the EPS and EDTA-Na in a dose-dependent manner, and the ferrous ion chelating effects of all the purified fractions (EPS-1, EPS-2 and EPS-3) were weaker than that of crude EPS and EDTA-Na. The metal chelating activity increased with increase in the EPS concentration up to 2.0 mg/mL, but further increases in samples concentration did not affect the activity significantly. The result is similar to those obtained by Qiao et al. (2009), which reported that ferrous ion chelating activities of different polysaccharide samples increased significantly with the increase of samples concentration ranging from 0.05 to 1 mg/mL. After that, the metal chelating activity increased not significantly with the increase of polysaccharide concentration. However, Xu, Shen, Ding, Gao, and Li (2011) reported that the metal chelating activity of the EPS isolated from *Bifidobacterium animalis RH* could increase with the increasing EPS concentration and reach to a similar activity with positive control. In this study, at the concentration of 4 mg/mL, the chelating power was 99.34%, 59.11%, 53.19%, 45.86% and 38.72% for Vc, crude EPS, EPS-3, EPS-2 and EPS-1, respectively.

The result reveals that the crude EPS, EPS-3 and EPS-2 demonstrate an effective capacity for ferrous ion binding, suggesting that its action as antioxidant may be related to its ferrous ion-binding capacity. As indicated in the literature, acidic polysaccharides are likely to contain more uronic acids and with negative charges (Ye et al., 2012). The atoms of polysaccharides with a greater proportion of uronic acid were negatively charged, resulting in more chelating activity when a metal ion attacks. This explains the reason for high content of uronic acid in acidic polysaccharides having higher metal ion chelating activity, especially for ferrous ion.

4. Conclusion

In the present study, the production, purification, characterization and antioxidant activities *in vitro* of EPS from *L. helveticus* MB2-1 were investigated. The maximum yield of EPS (658 mg/L) was obtained after 32 h. The crude EPS obtained from the reconstituted whey medium was purified by chromatography of DEAE-52 and Sephadex G-100, affording three fractions of EPS-1, EPS-2 and EPS-3. And characterization of the three fractions demonstrated that they were mainly composed of galactose, glucose and mannose with different molar ratios. In addition, the assay of the antioxidant activities *in vitro* demonstrated that both the crude and purified EPS had strong scavenging activities on three free radicals and metal ion chelating activity. And the antioxidant activities decreased in the order of crude EPS > EPS-3 > EPS-2 > EPS-1, according to the assays of scavenging activity on DPPH radical, hydroxyl radical and superoxide radical and chelating activity on ferrous ion. These results indicate that EPS from *L. helveticus* MB2-1 may be a new source of natural antioxidants with potential value for therapeutics and health food. And further works on the defined structure and potential application as an antidiabetic, anticancer and antiulcer agent are in progress.

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